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PRINCIPAL INVESTIGATOR: Paul Yaswen, Ph.D.

CONTRACTING ORGANIZATION: University of California at Berkeley
Berkeley, California 94720

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Paul Yaswen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of California at Berkeley
Berkeley, California 94720

E-Mail: P_Yaswen@LBL.GOV

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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

The failures to growth arrest in response to critically short telomeres or to TGFβ are key derangements thought to contribute to the inappropriate cell growth that characterizes breast cancer progression. Our recent observations indicate that activation of telomerase activity may be directly involved in overcoming both inhibitory pathways in human mammary epithelial cells (HMEC). By exploring the common thread connecting telomerase expression and resistance to TGFβ growth inhibition, it may be possible to unify two divergent areas of significance for breast cancer development and treatment. We have begun to dissect the complex mechanisms responsible for these changes in expectation that such knowledge may enable us to prevent or reverse them. We have found that in addition to abrogation of TGFβ-mediated growth arrest, high telomerase expression is also able to partially interfere with growth arrest due to blockage of EGF receptor signal transduction. The PI3K pathway appears to be necessary for this effect, and may either interact cooperatively with a telomerase influenced pathway or may act downstream of telomerase. We have examined several molecules involved in cell cycle regulation, and are presently exploring a promising new lead suggesting that telomerase may exert its effects indirectly through a p53-dependent pathway.

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INTRODUCTION

Finite lifespan human mammary epithelial cells (HMEC), like all normal human epithelial cells, ultimately growth arrest in the presence of transforming growth factor-beta (TGFβ). In contrast, immortally transformed and tumor-derived HMEC lines, like most immortal human epithelial cells, may proliferate indefinitely in the presence of TGFβ, although often at a slower rate. In our recent studies of HMEC immortal transformation induced by exposure to carcinogens or the breast cancer-associated oncogene, *ZNF217*, acquisition of endogenous telomerase activity was closely correlated with subsequent gradual acquisition of TGFβ resistance. Introduction of hTERT, the catalytic subunit of telomerase, into finite lifespan telomerase(-) HMEC lacking expression of the cyclin-dependent kinase inhibitor (CDKI) $p16^{INK4A}$, indicated that hTERT is the limiting component of telomerase activity, and is sufficient to produce an indefinite lifespan in these cells. Additionally, hTERT induced resistance to TGFβ-induced growth arrest. Induction of TGFβ resistance required catalytically active hTERT capable of extending telomeres in vivo. We hypothesize that high hTERT expression alters the abundance, modification, and/or spatial arrangements of signaling molecules mediating TGFβ-induced growth arrest. The induction of TGFβ resistance by hTERT in cultured HMEC, both through exogenous introduction and endogenous reactivation, may well model a key change occurring during human breast carcinogenesis *in vivo*. By exploring the common thread connecting telomerase expression and resistance to TGFβ-induced growth arrest, it may be possible to unify two divergent areas of significance for breast cancer development and treatment, and to provide new targets for therapeutic intervention in breast carcinogenesis.

BODY

Technical Objective 1: Determine whether reduced p27 levels alone can interfere with TGFβ-mediated growth inhibition of HMEC.

In our proposal, we noted a correlation between the ability of HMEC to maintain high levels of the CDKI p27 in the presence of TGFβ with the ability to exhibit growth inhibition. However, as reported previously, we learned at the June, 2000 DOD Era of Hope meeting from our collaborator that a p27 blocking experiment had already been performed and that antisense p27 expression was incapable of interfering with TGFβ-mediated growth inhibition in normal finite lifespan HMEC. In light of this development, we refocused our attention on different mechanisms that might mediate the growth inhibition. Another important aspect of the TGFβ anti-proliferative program is the inhibition of c-myc expression. C-myc is a ubiquitous transcription factor involved in cell proliferation. Levels of c-myc mRNA and protein in normal HMEC are decreased in response to TGFβ. We previously reported that fully immortal HMEC exhibited deregulated c-myc, and that c-myc levels were elevated in hTERT-transduced relative to control HMEC when both cell types were subjected to blockage of epidermal growth factor receptor (EGFr) signal transduction. However, in further studies, we were unable to demonstrate consistent differences in c-myc expression in our hTERT-transduced and control cells in the presence of TGFβ (Fig.1).

We have confirmed the striking finding that hTERT-transduced HMEC display elevated ability to continue to synthesize DNA in the absence of EGFr signal transduction. While this is true for hTERT-transduced cells expressing high levels of telomerase, it is not the case for HMEC expressing low levels of endogenous telomerase, and therefore may be related to the level of telomerase expressed. We are following up this observation in the expectation that information about this defect in growth control might also explain the loss of

TGFβ growth inhibition. To determine whether hTERT might simply increase levels of EGFr beyond those saturable by the anti-EGFr antibody used in blocking experiments, we performed an experiment using increasing concentrations of antibody. The hTERT transduced cells continued to exhibit statistically significant increased levels of DNA synthesis, negating this hypothesis (Fig.2). We have also examined downstream signal transduction elements for evidence of aberrant regulation or function. Our collaborator, Dr. Slingerland, has demonstrated that constitutive activation of the phosphotidal inositol-3-kinase/protein kinase B (PI3K/PKB) pathway alters p27 localization and function, and causes TGFβ resistance (1). We found that the labeling index of hTERT-transduced HMEC in the absence of EGFr function could be reduced when the cells were treated with low concentrations of the PI3K inhibitor, LY294002, suggesting that PI3K may either interact cooperatively with an hTERT-influenced pathway or may act downstream of hTERT (Fig.3). However, in our initial experiments, we could not detect significant differences in the levels of phosphorylated or total PKB (Fig.4), or in the levels of PI3K activity (data not shown) in hTERT-transduced vs. control cells. We also examined the expression of the active form of MEK-1, another downstream mediator of EGF signal transduction, and found no significant differences when the cells were deprived of EGF signaling.

The CDKI p21 is closely related to p27, and has been implicated in some cell types in growth inhibition due to both growth factor withdrawal and TGFβ exposure. In prior studies in collaboration with Dr. Slingerland, we did not observe changes in p21 association with cdk complexes after TGFβ exposure (2). However, total cellular p21 levels were not examined in these experiments, nor were sensitive and resistant cells directly compared. New preliminary experiments in our lab suggest that p21 may be transiently increased in control cells, but not hTERT-transduced HMEC after growth factor withdrawal (Fig.5). P21 levels then gradually decrease, while p27 levels gradually increase in both cell types. P21 transcription is known to be directly responsive to the p53 tumor suppressor, and p53 itself has recently been shown to be involved in growth arrest after serum withdrawal in human fibroblasts (3). We have performed preliminary experiments using a dominant negative genetic suppressor element to inactivate p53 in HMEC cultures. Interestingly, these experiments indicate that loss of p53 induces resistance to both TGFβ-induced growth arrest and growth arrest by blockage of EGFr signal transduction (data not shown). Induction of resistance to TGFβ-induced growth arrest is not immediate, and proceeds at different rates in hTERT-transduced normal and carcinogen-treated conditionally immortal HMEC, indicating an indirect effect. We are currently investigating the hypothesis that hTERT can interfere with signaling pathways upstream of p53, thereby inhibiting its activation in response to specific inducers.

Technical Objective 2: Determine how TGFβ-mediated stabilization of p27 is disrupted in hTERT expressing cells.

The majority of the tasks proposed for this objective have been obviated for the reasons stated above, and have been superseded by new tasks related to the overall goal which is to understand how hTERT expression confers resistance to TGFβ-mediated growth arrest. We have performed Task 7, the comparison of activated PKB levels in TGFβ-treated control and hTERT expressing HMEC. No significant differences were found in these experiments (Fig.6).

Technical Objective 3: Determine whether hTERT-induced resistance to TGFβ-mediated growth inhibition requires continuous telomerase activity and/or access to telomeres.

This objective has been completed. Please see previous progress report for details.

Technical Objective 4: Determine whether hTERT induces resistance to TGFβ-mediated growth inhibition in other HMEC types.

The first task, examination of other HMEC types, has been completed. The second task, examination of pathways which may differ in cells in which hTERT does or does not induce TGFβ resistance, will be performed once we have a better idea of which pathways mediate this effect.

KEY RESEARCH ACCOMPLISHMENTS

- hTERT-transduced HMEC show increased resistance to growth arrest by blockage of EGFr signal transduction.

- Low concentrations of the PI3K inhibitor, LY294002, mitigate the resistance of hTERT-transduced HMEC to growth arrest by blockage of EGFr signal transduction.

- No significant differences could be detected in the levels of phosphorylated or total PKB, or in the levels of PI3K activity in hTERT-transduced vs. control cells.

- Loss of p53 function, like hTERT transduction, can induce resistance to both TGFβ-induced growth arrest and growth arrest by blockage of EGFr signal transduction

REPORTABLE OUTCOMES

CONCLUSIONS

A high level of telomerase activity is one of the most common distinguishing features of cancer tissues and tumor-derived immortal cell lines when compared to normal human somatic tissues and finite lifespan cells. In the absence of high telomerase levels, replicative senescence halts cell proliferation before all the errors necessary for invasive cancer can accrue. Overall, our data indicates that in addition to its role in stabilizing telomeres, telomerase may have effects on growth control in susceptible cells. These effects may contribute to the growth autonomy of cancer cells. Exploring the mechanisms responsible for these effects will contribute not only to better understanding of breast cancer pathogenesis, but also to understanding of potential problems involved in the therapeutic use of telomerase to extend cell lifespan.

Thus far, we have found that in addition to abrogation of TGFβ-mediated growth arrest, high hTERT expression is also able to partially interfere with growth arrest due to blockage of EGF receptor signal transduction. The PI3K pathway appears to be necessary for this effect, and may either interact cooperatively with an hTERT-influenced pathway or may act downstream of hTERT. We have examined several molecules involved in cell cycle regulation, without yet uncovering a mechanistic link to hTERT alteration of growth control. We are

presently exploring a promising new lead suggesting that hTERT may exert its effects indirectly through a p53-dependent pathway.

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3. Itahana, K., Dimri, G. P., Hara, E., Itahana, Y., Zou, Y., Desprez, P. Y., and Campisi, J. A role for p53 in maintaining and establishing the quiescence growth arrest in human cells. J Biol Chem, *277:* 18206-18214, 2002.

APPENDICES

Fig.1 Total c-myc protein abundance is reduced to similar extents in control or hTERT-transduced HMEC treated with TGFβ. An immunoblot containing protein extracts from HMEC strain 184 transduced with control vector (LXSN) or hTERT incubated in complete growth medium (Cycl), medium minus EGF plus EGFr blocking antibody mAb225 (G0), or medium plus TGFβ (TGFβ) was incubated with antibodies to c-myc. Total c-myc protein abundance was then visualized by immunoperoxidase staining and chemiluminescence. Two different passage levels (12 and 15) of the hTERT-transduced cells were assayed.

Fig. 2 hTERT-transduced HMEC continue to show elevated DNA synthesis even in the presence of increased EGFr blocking antibody. Growing cultures of control (LXSN) or hTERT-transduced HMEC lines 184 and 161 were incubated in complete medium or were deprived of EGF and subjected to indicated concentrations of anti-EGFr antibody mAb225 for 48 hrs. with ^{3}H-thymidine added for the final 24 hrs. The labeling indices and standard deviations were determined by autoradiography in six representative fields for each sample.

Fig. 3 The PI3K inhibitor, Ly294002, reduces residual DNA synthesis in hTERT–transduced HMEC during blockage of EGFr signal transduction. hTERT-transduced 184 cells in complete growth medium (Cycling), or medium minus EGF plus EGFr blocking antibody mAb225 (G0 arrest) for 48 hrs. were subjected to indicated concentrations of Ly294002 with 3H-thymidine added for the final 24 hrs. The labeling indices and standard deviations were determined by autoradiography in six representative fields for each sample.

Fig. 4 Differences in the levels of total or phosphorylated PKB and MEK-1 do not correlate with the ability of hTERT-transduced HMEC to exhibit elevated labeling indices in the absence of EGFr signal transduction. Protein extracts of control or hTERT-transduced HMEC at the indicated passages in complete growth medium (Cycl.), or medium minus EGF plus EGFr blocking antibody mAb225 (G0) were probed with antibodies specific for the indicated proteins. Proteins were detected and labeling indices were determined as described above.

Fig. 5 Temporal changes in p21 and p53 after inhibition of EGFr signal transduction differ in control and hTERT-transduced HMEC. Protein extracts were made of control or hTERT-transduced 184 HMEC at the indicated timepoints after growth medium was changed to medium minus EGF plus EGFr blocking antibody mAb225. These extracts were then immunoblotted with antibodies specific for A) p21, B) p53, or C) p27. The resulting images were quantitated and normalized to the values for cells in complete growth medium at 0 hrs. When relative expression changes were plotted vs. time, the control cells showed transient increases in p21 and p53 at 12 hrs., while the hTERT-transduced cells did not. Both cell types showed gradual increases in p27 levels, although the magnitude of the increase was somewhat greater in the hTERT-transduced cells.

Fig. 6 Differences in the levels of total or phosphorylated PKB do <u>not</u> correlate with the ability of hTERT-transduced HMEC to grow in the presence of TGFβ. Protein extracts of control or hTERT-transduced 184 or 184A1 HMEC in complete growth medium ± TGFβ were probed with antibodies specific for total or

phosphorylated (activated) PKB. The proteins were detected as described above. The lane marked with an asterix was underloaded.

Figure 1

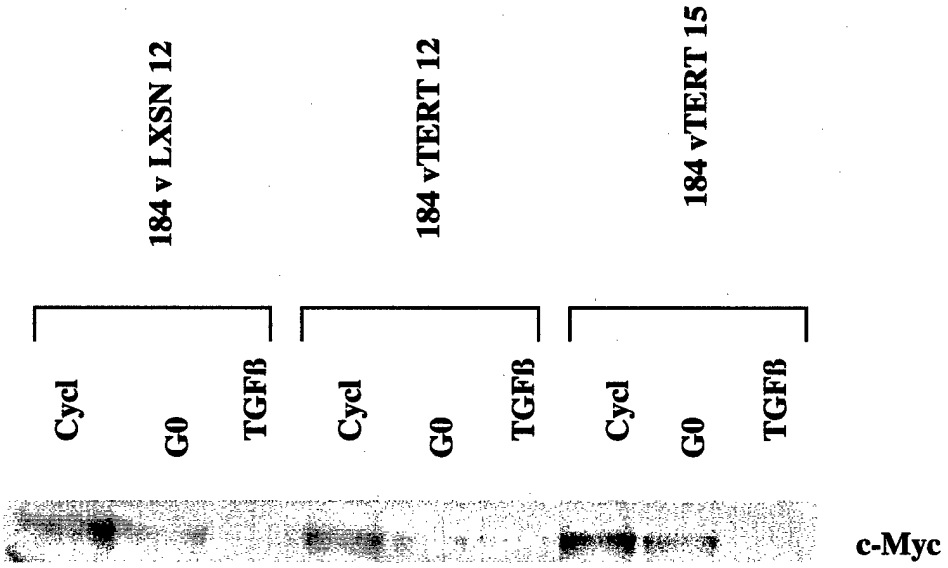
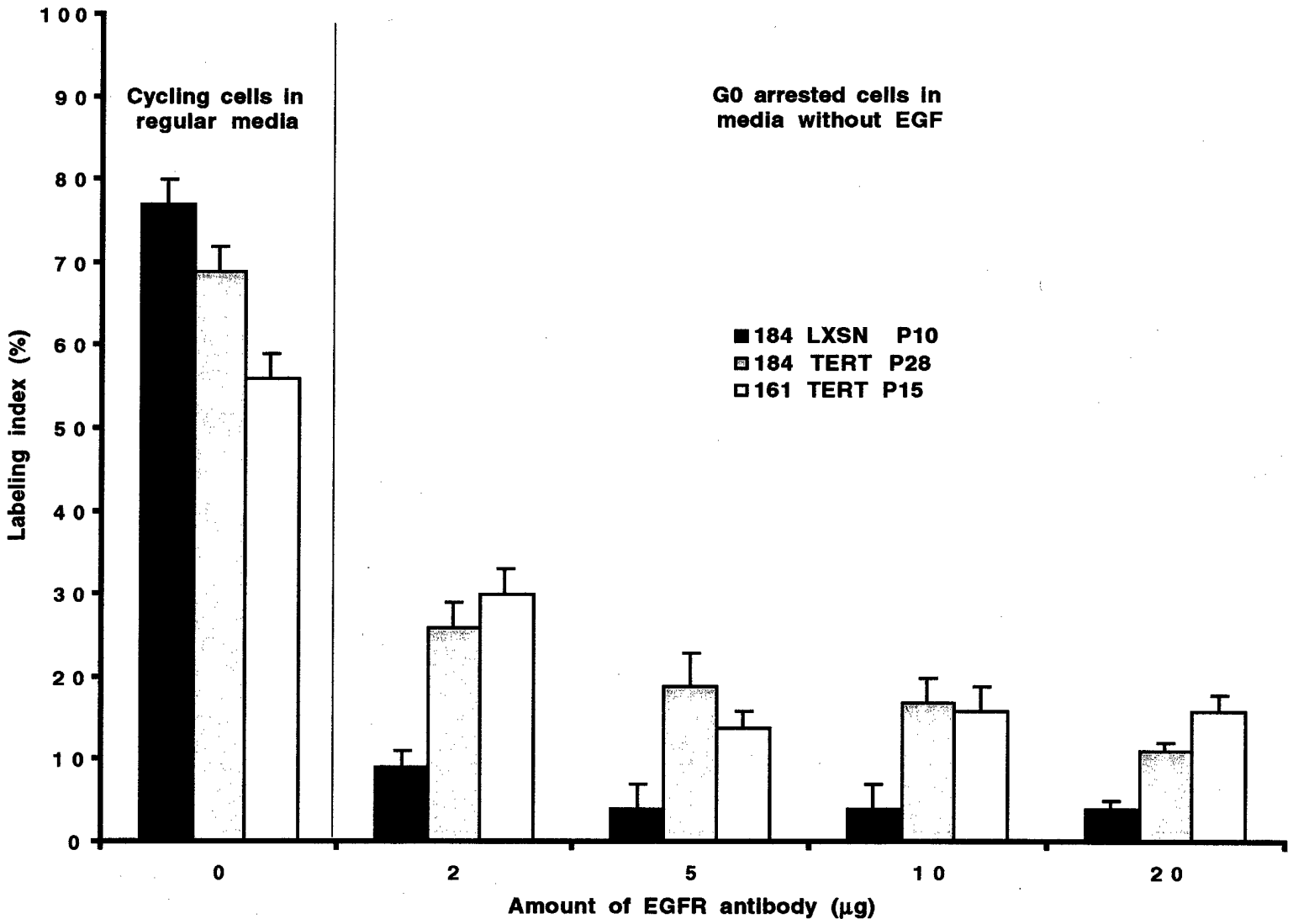

184 v LXSN 12
184 vTERT 12
184 vTERT 15
Cycl
G0
TGFß
Cycl
G0
TGFß
Cycl
G0
TGFß
c-Myc

Figure 2


100
90
80
70
60
50
40
30
20
10
0
Labeling index (%)
Cycling cells in regular media
G0 arrested cells in media without EGF
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184 TERT P28
161 TERT P15
0
2
5
10
20
Amount of EGFR antibody (µg)

Figure 3

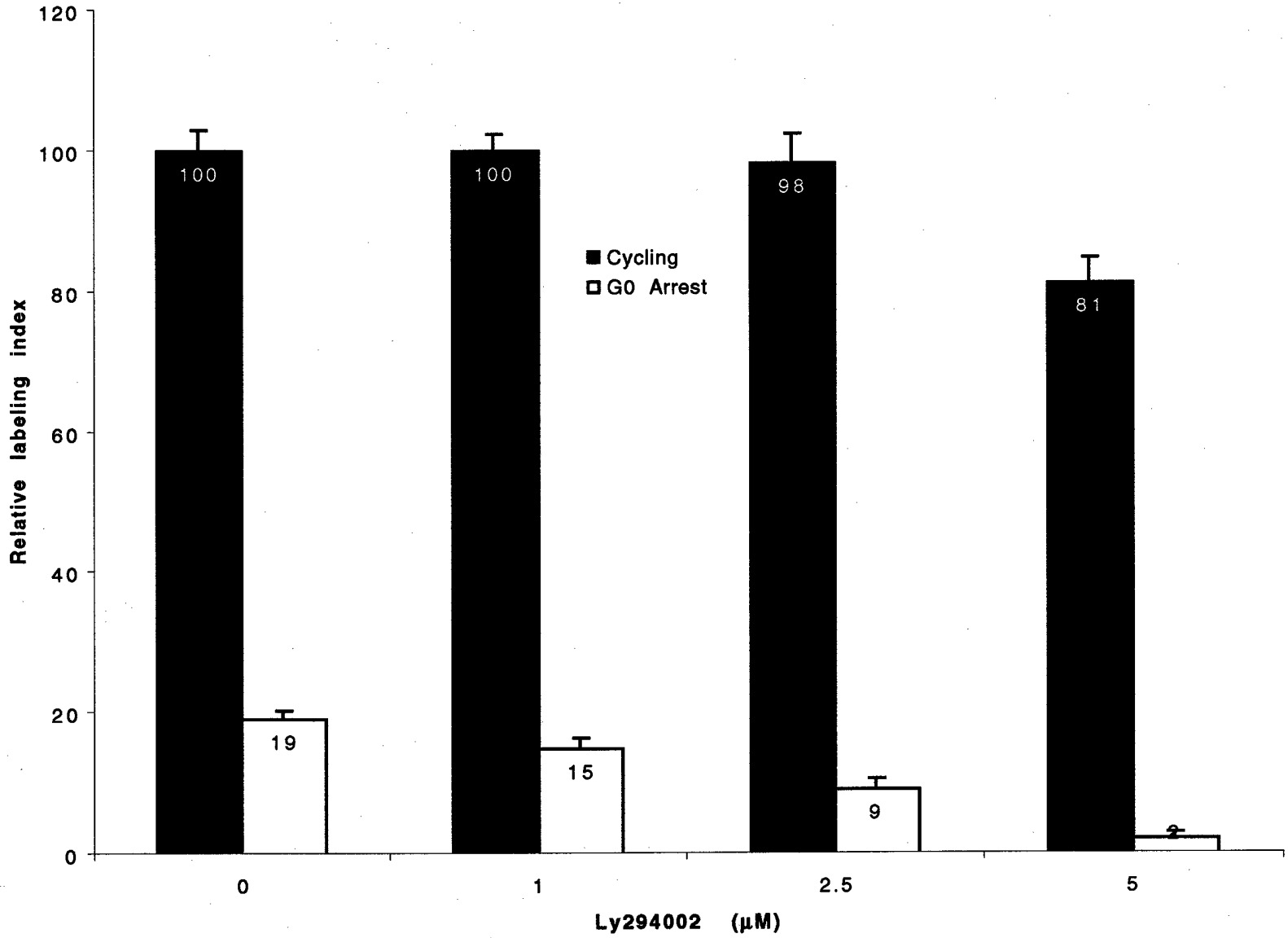
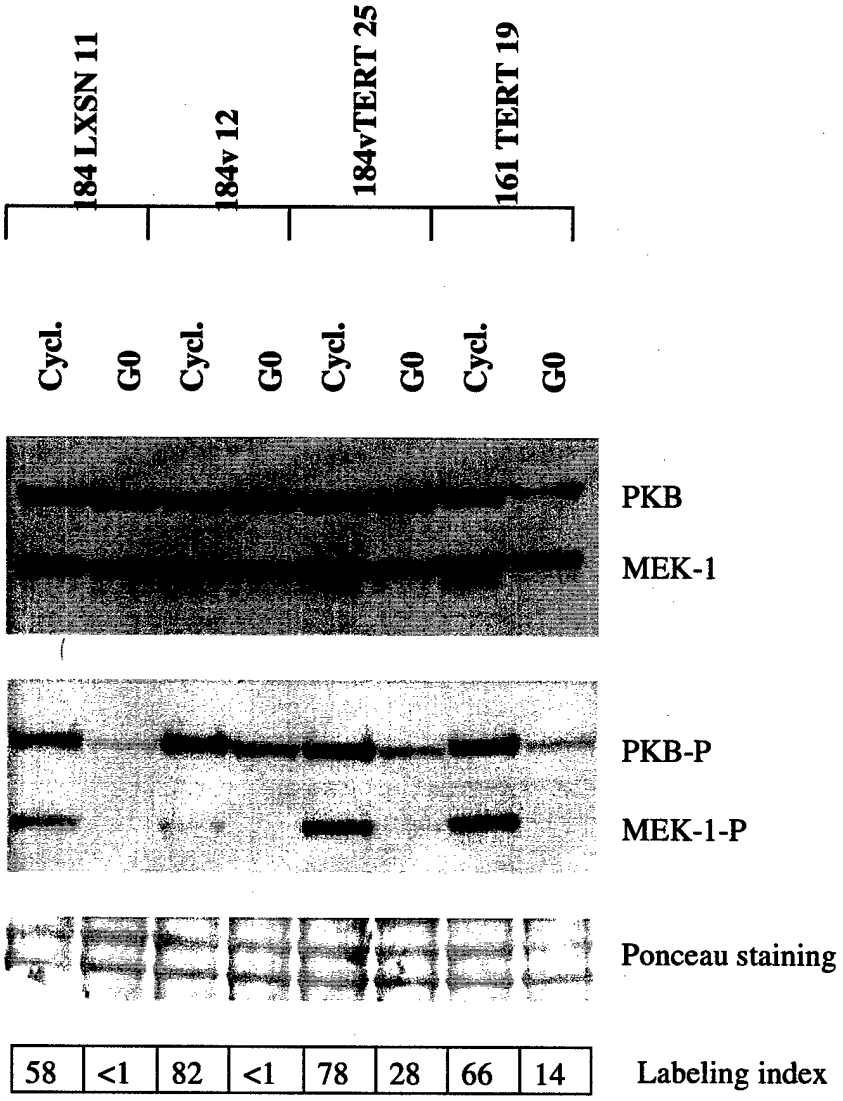

120
100
80
60
40
20
0
Relative labeling index
100
100
98
81
19
15
9
Cycling
G0 Arrest
0
1
2.5
5
Ly294002 (μM)

Figure 4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184v 12
184vTERT 25
161 TERT 19
Cycl.
G0
Cycl.
G0
Cycl.
G0
Cycl.
G0
PKB
MEK-1
PKB-P
MEK-1-P
Ponceau staining
58
<1
82
<1
78
28
66
14
Labeling index

Figure 5A

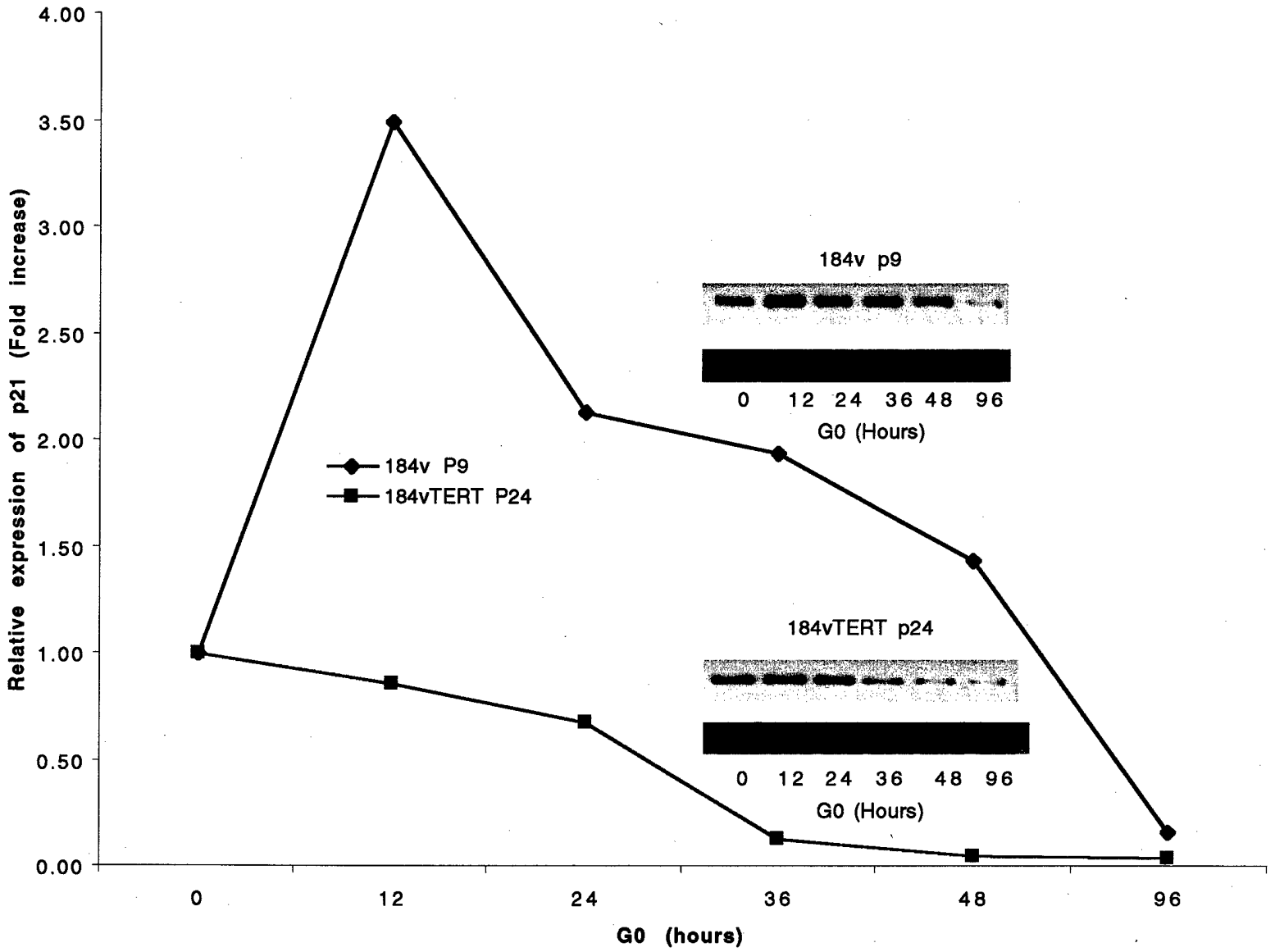

4.00
3.50
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Relative expression of p21 (Fold increase)
184v P9
184vTERT P24
184v p9
0 12 24 36 48 96
G0 (Hours)
184vTERT p24
0 12 24 36 48 96
G0 (Hours)
0
12
24
36
48
96
G0 (hours)

Figure 5B

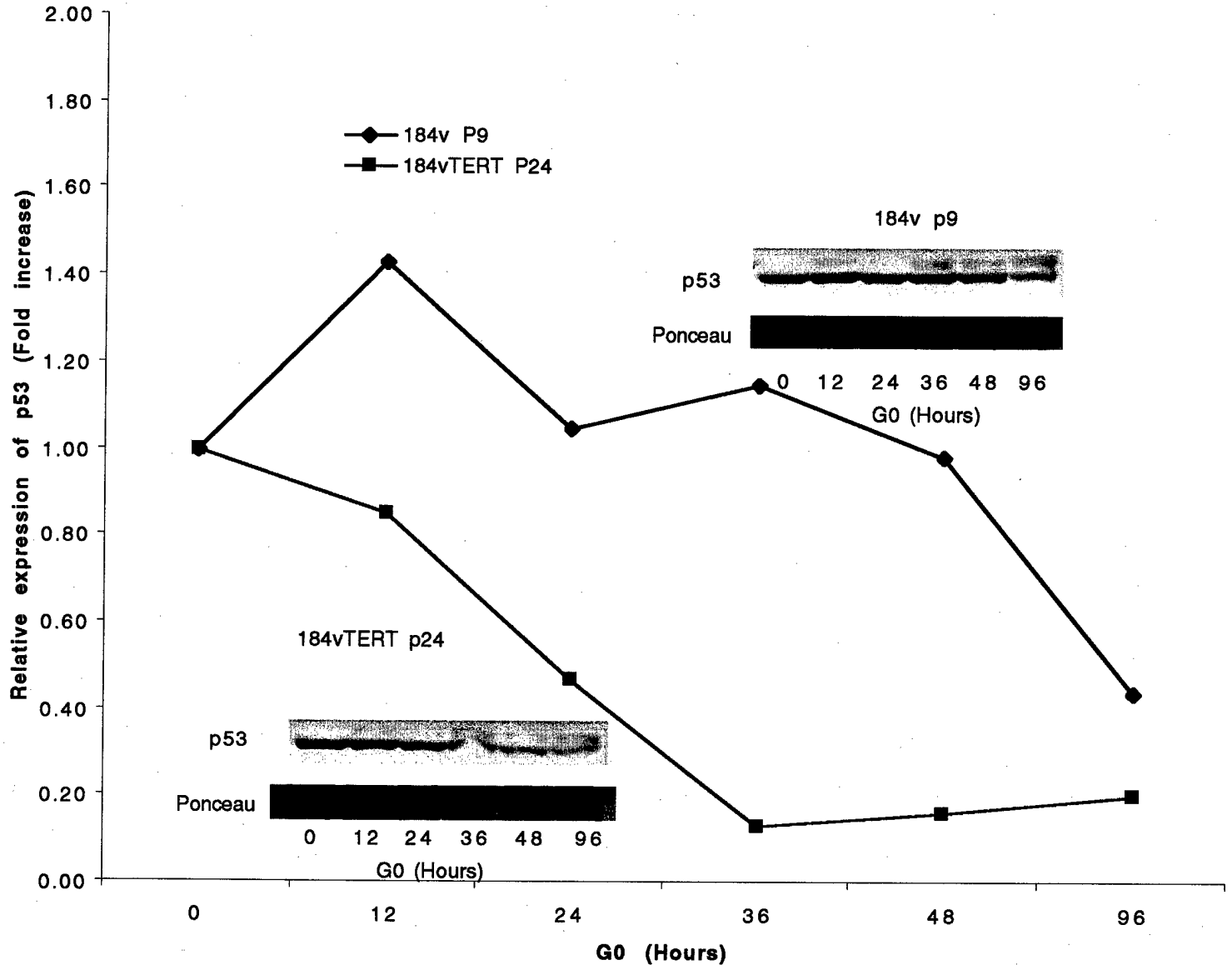

2.00
1.80
1.60
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
Relative expression of p53 (Fold increase)
184v P9
184vTERT P24
184v p9
p53
Ponceau
0 12 24 36 48 96
G0 (Hours)
184vTERT p24
p53
Ponceau
0 12 24 36 48 96
G0 (Hours)
0
12
24
36
48
96
G0 (Hours)

Figure 5C

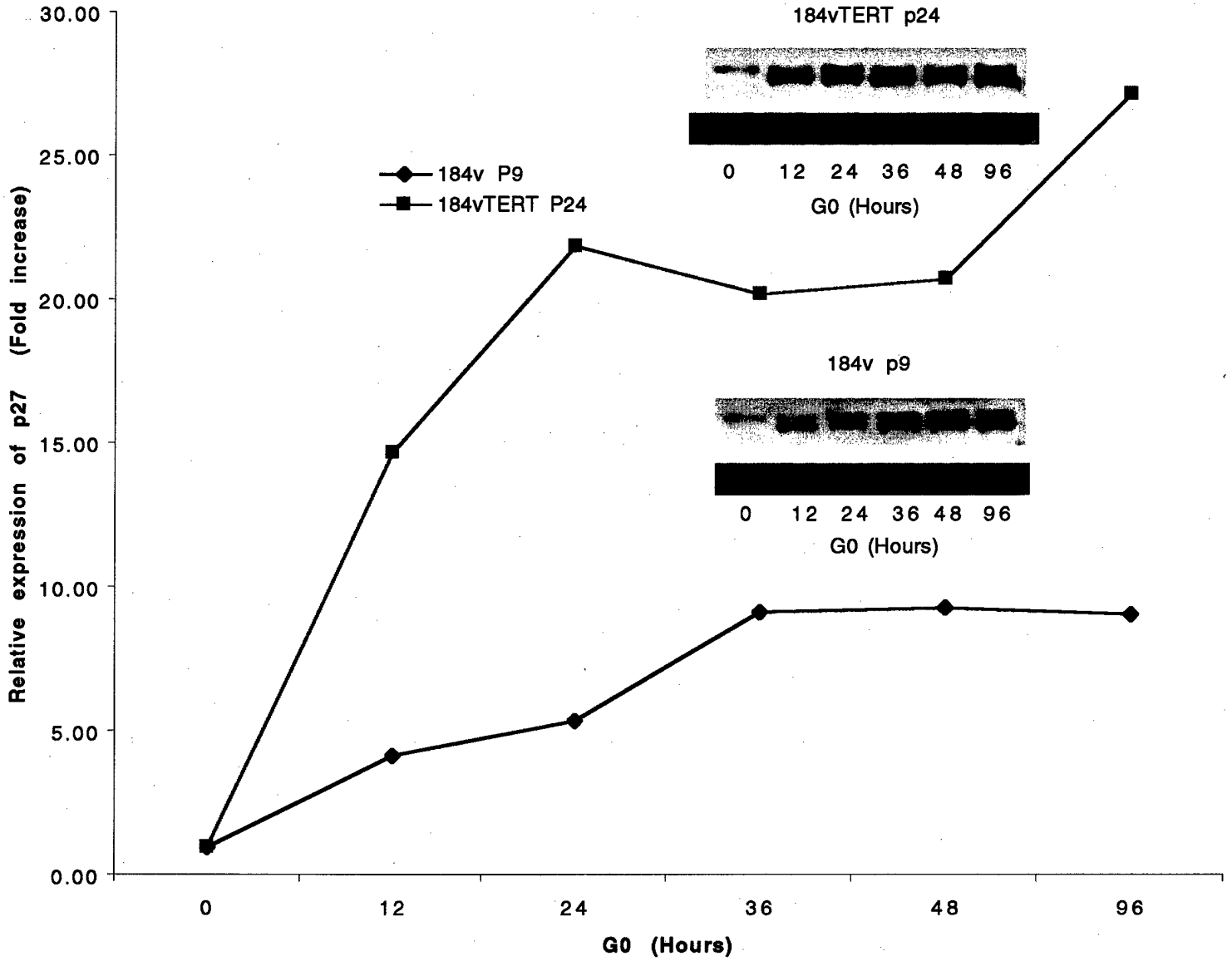
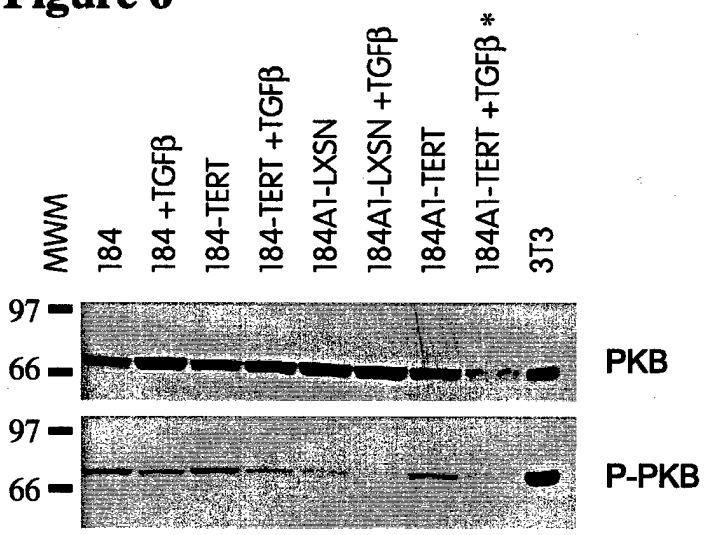

184vTERT p24
0 12 24 36 48 96
G0 (Hours)
184v P9
184vTERT P24
184v p9
0 12 24 36 48 96
G0 (Hours)
Relative expression of p27 (Fold increase)
30.00
25.00
20.00
15.00
10.00
5.00
0.00
0
12
24
36
48
96
G0 (Hours)

Figure 6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184
184 +TGFβ
184-TERT
184-TERT +TGFβ
184A1-LXSN
184A1-LXSN +TGFβ
184A1-TERT
184A1-TERT +TGFβ *
3T3
97
66
97
66
PKB
P-PKB